REMARKS

Claims 1-24 are pending and were all rejected under various grounds as discussed below.

Claims 1 and 24 are being amended almost identically. Claim 24 is also amended to remove a reference to "chromosome derived." Claim 24 was initially added without this limitation to distinguish it from claim 1, and Applicant wrote:

> New independent claim 24 has been added to remove the "chromosome-derived" language present in claim 1. Otherwise, it is identical to amended claim 1. Support for this claim can be found, inter alia, at page 5, lines 23-25 which read: "It is also very easy to determine the DNA concentration and hence the copy number of the nucleotide sequence per volume"

However, in the prior Response, where both claims were amended identically, this language was inadvertently added back. Applicant now corrects this error.

Amendments to claim 1 and 24 are supported throughout the specification. Specific support is indicated below in the relevant parts of the discussion. No new matter is added, and the entry of these amendments and remarks is respectfully requested.

I. Withdrawn Claim Rejections

Applicant thanks the Examiner for his withdrawal of rejections under § 112, 2nd paragraph as enumerated in sections 4-11 of the Action

II. Art Cited by Applicant Not Provided

In Sec. 12 of the Action, the Examiner noted that the Office did not receive references discussed in papers filed on 11/02/2007 and 05/30/2008, now a new Information Disclosure Statement. Hence those references were not considered.

Applicant's Response

However, the undersigned inadvertently failed to include these documents, and no IDS submitted to make them officially of record. Such and IDS, and the references, are submitted herewith. The first was discussed in the last Office Action, whereas the latter two are discussed herein.

1. Reischer *et al.* (2007) Lett. Appl. Microbiol. 44:351-356 (discussed in prior Response)
2. Winer, J. *et al.* Analytical Biochemistry, 270:41-49 (1999).
3. Georgieva, SG. *et al. Molecular Biology*, 35:961-964 (2001).
   Transl from *Molekulyarnaya Biologiya*, 35:1116-1119 (2001).

III. Rejections Under 35 U.S.C. § 112, First Paragraph (Enablement)

The rejection of claims 1-24 for lack of enablement was maintained. The Office contended that the specification is enabling only for detection using probes with both quenchers

and fluorophore. Allegedly, the specification does not reasonably provide enablement for detection by a fluorophore alone by any measure of concentration.

A Wands analysis was provided by the Office and the Office's points are incorporated into the discussion below

Applicant's Response

Applicant respectfully traverses this ground for rejection

The skilled artisan would fully understand and know that when using the claimed method, as is common practice with RT-PCR reactions, a fluorescently-labeled probe means a probe comprising a fluorophore and a quenching moiety, often via FRET (fluorescence resonance energy transfer). For discussion of fluorescently labeled probes as used herein, see, for example, Figure 1A or 1B of Ginzinger *et al.* (2002) *Exp. Hematology 30*:503-12 (of record).

However, for greater precision, and to overcome the rejection, Applicant is replacing "fluorescently-labeled probe"" with "probes comprising a fluorophore and a quencher" (in claims 1 and 24)

Nature of the Invention and Breadth of Claims

Although a typical TaqMan probe requires 5'-3' exonuclease activity (Ginzinger, *supra*, page 505, Figure 2A legend), not all amplification techniques using a probe comprising a fluorophore and a quencher require exonuclease activity. Ginzinger (*supra*) also teaches that a Molecular Beacon type of probe is not cleaved by the 5'-3' exonuclease activity and only generates signal when hybridized to the template (see page 505, legend to Figure 2B, right col.). Therefore, exonuclease activity, although sometimes required, *e.g.,* when typical TaqMan probes are used, should not be considered a necessary feature of the claims.

Please note that Zhang *et al.* (1997) *Biochem. J. 321*:769-75 and Zhang *et al.* (1999) *Biochem. J. 337*:231-41 (both of record) do not use a probe in their technique. Their method rather relies on non-specific intercalation of Ethidium Bromide (EtBr) between the base pairs of the nucleic acid amplification product. The longer the amplification product is, the more EtBr can intercalate and the brighter the fluorescence signal. Therefore one reason that EtBr (and this also applies for SYBRgreen technology) cannot be used in the presently claimed method is that the fluorescence signal differs in intensity in proportion to the length of the nucleic acid fragment. Thus, use of EtBr requires that there be a length difference of the nucleic acid fragment, since (i) separation on a gel is the only way to discriminate the fragments and (ii) the fragments are produced in a single container. As stated above, The two Zhang references do not

use a probe. Therefore, they are outside the scope of the present claims so that enablement should not be an issue.

In contrast to the methods of Zhang *et al.* (1997 and 1999, *supra*) (and also to methods based on SYBRgreen technology), in amplification methods that employ a probe to one nucleic acid fragment to be amplified, only one probe can bind. This results in a proportional relationship of the amount of fluorescence released and the number of amplified copies that (*independent* of length) of the nucleic acid amplification products that are present. Therefore, an amplification technique for *more than one* nucleic acid amplification product using probes comprising a fluorophore and a quencher specific for the nucleic acid products (*e.g.*, RT-PCR) can be carried out *in a single container* using different fluorophores for the probes for the different nucleic acid products (Ginzinger, *supra*, pg. 504, 4th full paragraph, especially the last 4 lines of page 504. At page 8 of the specification, a probe for NucSeqI has the fluorophore FAM (line 7) and the probe for NucSeqII has the fluorophore TexasRed as (line 15)).

Applicant is amending claims 1 and 24 to include the feature that the first fluorophore and the second fluorophore are different.

Since amplification techniques for more than one amplification product in one container were known to the skilled artisan and are explained in detail, *e.g.* in Ginzinger *et al.* (2000) *Cancer Res 60*:5405–9 and Ginzinger, 2002 (*supra*), this person is enabled to practice the invention as claimed.

Determining Relative Copy Number Using Concentration (wt/vol)

To demonstrate that Conc-I_{SCI} and Conc-II_{SCI} need not be expressed in ng/volume, mole/volume or so on, Applicant offers the following examples. These used here only for explanatory purposes and are not intended in any way to limit the scope of the claims.

Example A

In standard curves SC_I and SC_{II}, dilution of NucSeqI' and NucSeqII' may be plotted against the Ct (threshold cycle). By amplifying NucSeqI and NucSeqII, the Ct of NucSeqI and the Ct of NucSeqII are measured. Since one nucleic acid sequence will only bind one probe, a difference in length of the nucleic acid sequence does not matter; as a consequence a difference in weight of the nucleic acid sequence for the amount of fluorescence generated does not matter.

The corresponding dilutions of NucSeqI' and NucSeqII' can be read from the standard curves SC_I and SC_{II}.

For this example let us assume that

(1) for the Ct of NucSeqI the corresponding NucSeqI' dilution is 8-fold;

(2) for the Ct of NucSeqII the corresponding NucSeqII' dilution is 6-fold;

(3) the ratio of concentration of NucSeqI' to the concentration of NucSeqII' is 3 (since the ratio of the concentration is known; see claim 1(2)(i)).

It then follows that:

- Conc-I_{SCI} is the ratio 1/8 to 3 = 0.041667
- Conc-II_{SCI} is the ratio 1/6 to 1 = 0.16667.

According to the formula of claim 1, the Relative CN = 0.041667/0.16667 = 0.25.

Example B

Here let us use wt/vol (*e.g.*, ng/ml) in the standard curves. For example for the Ct of NucSeqI the corresponding NucSeqI' weight is 0.6 ng/ml and for the Ct of NucSeqII the corresponding NucSeqII' weight is 0.8 ng/ml

Further, let us assume that the NucSeqI' concentration in the stock solution is 3 ng/ml and the NucSeqII' concentration in the same is 4 ng/ml (this follows from claim 1(2)(iv): NucSeqI' and NucSeqII' are localized on a single vector. In addition, since NucSeqI' and NucSeqII' are known, it is also known that NucSeqI' has a length of, **e.g.,** 0.6 kb and NucSeqII' has a length of, *e.g.*, 0.4 kb. The skilled artisan knows how to use these data to calculate the molar concentrations of NucSeqI' and NucSeqII' using the following **(known)** formula:

$$\text{Conc. of nucleic acid [mole/liter]} = \frac{\textit{conc of nucleic acid [grams/liter])}}{\text{(mean MW of one pair of nucleotides [Da] x length of nucleic acid [kb] x } 10^3\text{)}}$$

where the mean MW of a base pair is 649Da.

The above formula is from the website http://molbiol.edu.ru/eng/scripts/h01_07.html. Georgieva *et al.* (2001) *supra* (submitted herewith) describes the website and that the website facilitates conversion of weight concentration into molar concentration for nucleic acids (page 962, right column bullets 5 and 6. Thus, this website and the calculation tool for conversion of weight concentration into molar concentration existed before the present priority date (26 July 2002).

However, since the ratio of the molar concentrations is needed, this formula can be simplified so that the ratio of the molar concentration of NucSeqI' and NucSeqII' is easily calculated as:

$$\frac{concentrationNucSeqI'[grams/liter]}{lengthNucSeqI'[kb]} : \frac{concentrationNucSeqII'[grams/liter]}{lengthNucSeqII'[kb]}$$

Using this formula the ratio of the molar concentration of NucSeqI' : NucSeqII' in the stock solution is: (3/0.6) : (4/0.4) = 0.5

Since the lengths of both NucSeqI and NucSeqII are also known, a similar formula can be used to determine the relative copy number. For the ease of this discussion, let us assume that the length of NucSeqI = 0.6 kb and the length of NucSeqII = 0.4 kb.

$$\text{Relative CN} = \frac{concentrationNucSeqI[grams/liter]}{(lengthNucSeqI[kb]\times x)} : \frac{concentrationNucSeqII[grams/liter]}{(lengthNucSeqII[kb]\times y)}$$

wherein molar ratio of concentration of NucSeqI' : NucSeqII' = x : y.

Thus, using this formula, the relative CN = (0.6/(0.6 x 1) : (0.8/(0.4 x 2)) = 1

The above examples demonstrate that the use of concentration (wt/vol) in the claimed methods is enabled by the specification and the state of the art. Further, the foregoing also demonstrates that using only weight (ng) in the claimed methods is also enabled. The difference from the first example in the Office Action is that the Examiner seems to take the position that only the concentration is known, and, as a consequence, only the relative weight is known. However, this is not correct. When performing an amplification reaction, a skilled artisan knows the length of the NucSeqI', NucSeqI, NucSeqII' and NucSeqII, or else these lengths can be readily determined. Therefore, at the time the invention was made, the skilled artisan would have been able to carry out the calculations as shown above.

Remarks at Page 7 of the Office Action

Although it is not required that NucSeqI and NucSeqI' have the same length, it is not intended that NucSeqI can hybridize to two separate regions of NucSeqI' each of which has the same (or partially the same) nucleic acid sequence. In other words, it is not Applicant's intent, and the claims do not require that two copies of NucSeqI are able to bind simultaneously to a single copy of NucSeqI' (and vice versa). The same applies for NucSeqII - NucSeqII' relationship.

However, it should be understood that the single vector comprising NucSeqI' and NucSeqII' may comprise more than one copy of NucSeqI' and/or NucSeqII', *e.g.,* 2 copies of NucSeqI' and 1 copy of NucSeqII'.

Claims 1 and 24 have been similarly amended by adding the language (supported at page 4, lines 3-5):

"if NucSeqI and NucSeqI' differ in length, the shorter of the two is at most 30% shorter than the other;"

and

"if NucSeqII and NucSeqII' differ in length, the shorter of the two is at most 30% shorter than the other;"

Applicant contends that a skilled artisan will readily appreciate that the probes for NucSeqI, NucSeqI', NucSeqII and NucSeqII' need to be unique within the NucSeq, since that person is familiar with probe-based amplification techniques

It will it also be clear to the skilled artisan that a probe needs to be used for each of NucSeqI, NucSeqI', NucSeqII and NucSeqII'.

It is evident without further description that the probes for NucSeqI and NucSeqI' may be the same probes, with the same fluorophores and the same quenchers. Likewise, the probes for NucSeqII and NucSeqII' may be the same probes, with the same fluorophores and the same quenchers. As discussed above, the person of ordinary skill in the art will readily appreciate that at least the probes used for the pair of amplification reactions performed in a single container must have different fluorophores. Since probe-based amplification techniques are well known to those skilled the art, no undue experimentation would be required to practice the present claims to the full extent of their scope, so that the claims are fully enabled.

With respect to Gibson *et al.* (1996) ("Gibson") paper cited by the Office, Applicant notes that the target and internal control (that is coamplified with the target sequence within the same sample) can be compared to NucSeqI and NucSeqII, when they are measured in a single container.

In the situation described in Gibson, the target is directly compared with the internal control and in that case, it is very much preferred that the amplification conditions be highly similar for both the target (let us say NucSeqI) and the internal control (let us say NucSeqII). Gibson provides examples of such these amplification conditions that should be at least similar if not the same: the target and the internal control should use the same primers, have similar GC content and be of equal or similar length.

As can be understood from the present invention, there is no need for NucSeqI and NucSeqII to use the same primers, since these two sequences may be quite different. Therefore, any imposition by the Office requiring use the same primers would unjustly limit the scope of the present claims.

Further, the GC content does not necessarily have to be similar and length may vary, since the use of NucSeqI' and NucSeqII' according to the present invention will correct for differences in amplification conditions.

Thus, since Gibson relies on a different method of using control sequences in which concentration (as wt/vol) alone may be not sufficient to determine copy number, Gibson's teaching is not appropriate evidence that wt/vol concentration is insufficient to determine the relative copy number in the presently claimed method. In fact, the relative copy number can be determined from wt/vol concentrations alone, without any knowledge of the relationship of the

weight of a oligonucleotide to the number of copies present, as has been demonstrated in Example B above.

Working Examples and *Guidance in the Specification*

With respect to the discussion of these aspects of the Wands analysis, in the Action, it is believed that the present amendments and the foregoing remarks are sufficient to indicate the basis by which the claims are enabled. As stated above, Ginzinger (2002, *supra*) provides a disclosure that multiple fluorophores can be measured real-time in a single container. In addition, Examples I and II in the specification provide support for claim 1 as amended. As also noted above, the claimed method does not require a separation step as in Zhang *et al.* (1997) *supra*.

Unpredictability of the Art and State of the Prior Art

As for *Reliability*, Applicant believes that the present amendment, in light of the above remarks are adequate to satisfy this prong.

As for *Normalization*, in Example A and B above, Applicant has tried to illustrate how the essential steps/elements are present in the amended claims, as enabled by the specification and state of the art, to determine relative copy number. The skilled artisan would readily understand that the probe should be designed in such a way that the specificity and probe hybridization are similar for both NucSeqI and NucSeqI' (and, correspondingly, for NucSeqII and NucSeqII'). Furthermore, the skilled artisan would not use more than one label per probe. It a long-standing legal principle that a patent need not teach what is already well-known in the art.

Quantity of Experimentation

The skilled artisan knows that for the amplification reactions of the present claims, the probes must be labeled. Furthermore, it is well-known which fluorescent labels can be selected. (See, *e.g.*, Experiment I in the specification).

The claims state that at least one pair of amplification reactions (a) and (b) or (c) and (d) must be performed in a single container and is monitored by fluorescence during amplification. Therefore, it is evident to the skilled artisan that he can perform the claimed method in

(1) a competitive format - amplification reactions (a) and (b) are performed in a single container and amplification reactions (c) and (d) are performed in a single container) or

(2) a partly competitive format - only one pair of amplification reactions (a) and (b) or (c) and (d) are performed in a single container.

The skill, effort, and amount of and experimentation that might be needed to practice the methods are no greater than those needed for practicing any standard RT-PCR. And the

expectation that the claimed method can be performed with success is no less. There is thus no burden of undue experimentation demanded of the person of ordinary skill in the art.

Guidance in the Specification

Applicant believes that the amendments and foregoing remarks are adequate to overcome this prong.

Level of skill in the art (page 12)

The experimentation is needed for practice of the present claims (*e.g.,* to determine what NucSeqI and NucSeqII should, possibly creating a vector comprising NucSeqI' and NucSeqII', the design of primers, the design of probes comprising a fluorophore and a quencher, the carrying out amplification reactions, generation of standard curve, *etc.*, are all conventional and common skills in the field of molecular biology or molecular microbiology, particularly in the practice of amplification reactions. Therefore, the level of skill in the art needed to practice the claimed invention is commensurate with that possessed by the ordinarily skilled artisan in this field.

In view of the amendments and foregoing remarks, it would be appropriate to withdraw the pending rejection for lack of enablement.

IV. Rejections under 35 U.S.C. § 112, 2nd Paragraph

Claims 1-24 were rejected as being indefinite for several reasons, provided below, both based on the Office's contention that the claims are deficient due to incompleteness and omission of essential structural cooperative relationships of elements,

The Office Action contends the omitted structural cooperative relationships are:

(a) the relationship of the fluorescently labeled probes to NucSeqI, NucSeqII, NucSeqI', and/or NucSeqII' (independent claims 1 and 24; dependent claims 2-23)

(b) the step(s) of amplification "monitored by flourescence" which allow "determining from the results of amplification of step (2) the concentration of NucSeqI and NucSeqII' as recited in claims 1 and 24 and how such amplification, "monitored by fluorescence" relates to the standard curve of SC_I and SC_{II}.

Applicant believes that rejection (a) above is moot in view of the amendments.

As to rejection (b), Applicant disagrees that any step is omitted. It is clear to the skilled artisan that from at least two reference points generated by amplification of NucSeqI' and NucSeqII', respectively, at multiple dilutions and by monitoring the fluorescence during amplification, standard curves SC_I and SC_{II} can be generated. See for example the standard curves in Figures 1 and 2 of the application. The threshold cycle is the cycle at which

fluorescence starts to increase, as is known from the prior art. See, for example, e.g. Winer *et al.* (1999) *Anal. Biochem. 270*:41-9 (submitted herewith and discussed in the specification, at page 43 and Figure 1).

By determining the Ct of NucSeqI and NucSeqII in the unknown sample, the skilled artisan can easily read the logarithm of the starting quantity and copy number. See also Examples A and B presented above for further illustration.

Applicant should not be required to limit the generation of the standard curves to the Examples. As disclosed in the specification (page 3, lines 25-30) computational methods can also be used without the need to actually create a graph/curve. Thus, "making a standard curve" involves any method that relies upon at least two reference points to determine a relative concentration, *e.g.*, the log of the starting quantity, copy number, mass, or dilution.

In fact, Conc-I_{SCI} and Conc-II_{SCII} may be defined more broadly than just concentrations, since, from the above Examples, it is readily understood that mass (ng), which can of course be calculated from concentration, may be used instead of concentration in the standard curves. Most importantly, NucSeqI' and NucSeqII' are located on the same vector.

V. CONCLUSION

In view of the amendments to the claims and the foregoing remarks, Applicant believes that they have overcome or mooted the various grounds for rejection. Reconsideration, withdrawal of the rejections and allowance of the amended claims are respectfully requested.

The Examiner is respectfully requested to contact the undersigned at (202) 628-5197 if any clarification is required or if further discussion will assist in continued examination of this application

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